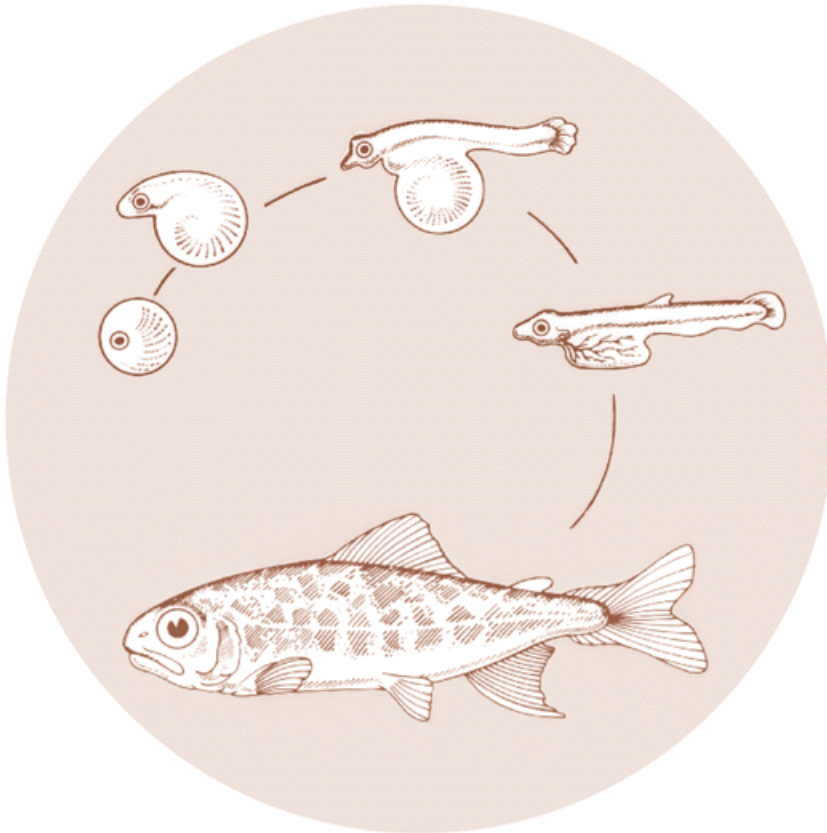


# EVALUATION OF A SUBUNIT VACCINE TO INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS

Report Period:  
July 31, 1988 to September 30, 1989

Annual Report



DOE/BP-16479-3



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# EVALUATION OF A SUBUNIT VACCINE TO INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS

Annual Report  
July 31, 1988 to September 30, 1989

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## INTRODUCTION

A recombinant DNA vaccine to IHNV was prepared and tested in field trials at Clear Springs Trout Company's Box Canyon Hatchery in Buhl, Idaho this year in Phase III of the project. The vaccine under consideration in these field trials consisted of lysed bacteria that contained a plasmid which expressed an antigenic portion of the IHNV glycoprotein. In addition, laboratory trials with a bacterial expressed viral nucleoprotein indicated that this served as an immune adjuvant. Therefore, a decision was made to conduct these field trials on a vaccine containing both IHNV glycoprotein and IHNV nucleoprotein.

Original plans to conduct the field trial at Dworshak National Fish Hatchery were canceled because a management decision was made by Dworshak Fish and Wildlife personnel to rear steelhead salmon eggs from IHNV positive parents at Kooskia National Fish Hatchery. This decision, which was made without prior notification to us, resulted in some discussion at the IHNV committee meeting convened by the Fish and Wildlife Service in Moscow, Idaho on April 27, 1989. At that time, our dismay at this decision was voiced and the prediction that an outbreak of IHNV would occur at Kooskia was made. In less than a week, a massive IHNV outbreak did occur at Kooskia and plans to run a field trial at this facility had to be discarded. An alternative site was found at the Box Canyon Hatchery site of Clear Springs Trout Company. Dr. Robert Busch, Director of Research and Development for Clear Springs Trout Company, offered us the use of the site. In preparation for the site change we consulted Mary Buckman, Oregon Department of Fish and Wildlife statistician, and we obtained a sample of the IHN virus present at Box Canyon.

The Box Canyon virus isolate was typed by reactivity with monoclonal antibodies by Dr. Sandra Ristow at Washington State University. There was insufficient time to examine the vaccine efficacy with the Box Canyon virus isolate in laboratory trials and we had to prepare for field trials without this supporting data. In addition, we had to make numerous changes in the vaccination schedule and in the design of the challenge to accommodate the new site. Most importantly, an application for a change in site had to be approved by the Idaho State Veterinarian and the Veterinary Biologics Group at the USDA National Office in Hyattsville, Maryland.

A new work plan was formulated, approvals were obtained, the demands for statistical analyses were satisfied in the new work plan, and 20,000 rainbow trout fry were vaccinated on July 19, 1989. The following is a summary of the results of the work that was initiated at Box Canyon Hatchery.

REVISED WORK PLAN FOR FIELD TRIAL  
OF  
IHNV VACCINE AT BOX CANYON HATCHERY  
BUHL, IDAHO'

. This is a copy of the revised work plan that was submitted to BPA in lieu of the original work planned at Dworshak National Fish Hatchery. It has been included in the annual report for completeness.

May 26, 1989

II. REVISED WORK PLAN - 1989  
CLEAR SPRINGS TROUT COMPANY, BOX CANYON HATCHERY  
BUHL, IDAHO

Objective 4. Field Trials.

To determine the efficacy of a recombinant subunit vaccine to IHNV in field experiments at Box Canyon Hatchery, Buhl, Idaho.

Task 4. Obtain approval for change of site for testing the vaccine from the following offices:

Subtask 4.0.1. Dr. W. Greg Nelson, Idaho State Veterinarian at P.O. Box 7249, Boise, Idaho 83707. Phone (208) 334-3256.

Subtask 4.0.2. Dr. George Shibley, U.S.D.A., Veterinary Biologics Staff, APHIS, VS, 6505 Belcrest Rd., Hyattsville, Maryland 20782. Phone (301) 436-8245.

Subtask 4.0.3. Notify Kent Hauck and Keith Johnson, Idaho Fish and Game, Eagle Fish Health Lab., Route 1, Trout Road, Eagle, Idaho 83616. Phone (208) 939-2413.

Task 4.1. Isolate IHNV from moribund fish at Box Canyon Hatchery.

Subtask 4.1. Take moribund fish from Box Canyon Hatchery to Corvallis, Oregon. Isolate virus using standard procedures.

Subtask 4.1.2. Prepare virus stocks for shipment to Drs. Robert Busch and Sandra Ristow.

Subtask 4.1.3. Prepare virus stocks for virus challenge studies.

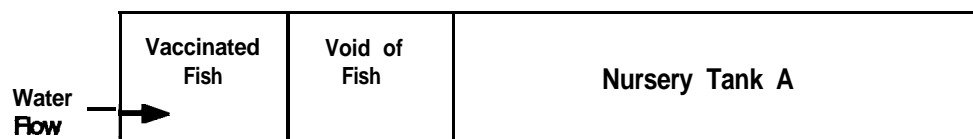
Task 4.2. Vaccinate IHNV negative fish at 1 .O g/fish (from a single egg lot) in the nursery building at Box Canyon Hatchery.

The fish will be vaccinated by immersion in a large holding tank in 20,000 fish groups. They will receive 0.75 mg of total protein (vaccine lysate) per fish or 15 g of protein per 20,000 fish. There will be a total of 200,000 fish in the vaccinated fish group. The control, unvaccinated group will receive no treatment and will be held in a separate raceway in the nursery. A detailed description of the procedures which will be followed is attached.

## TENTATIVE SCHEDULE

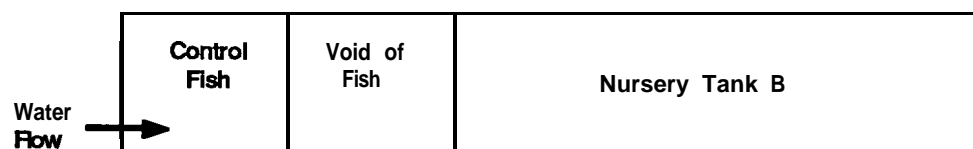
### Immunized Fish

Day 0 Vaccinate fish  
1 g size (400-450/lb)  
Raceway A in Nursery  
Even numbered raceways  
(Total: 40,000)



### Control Fish

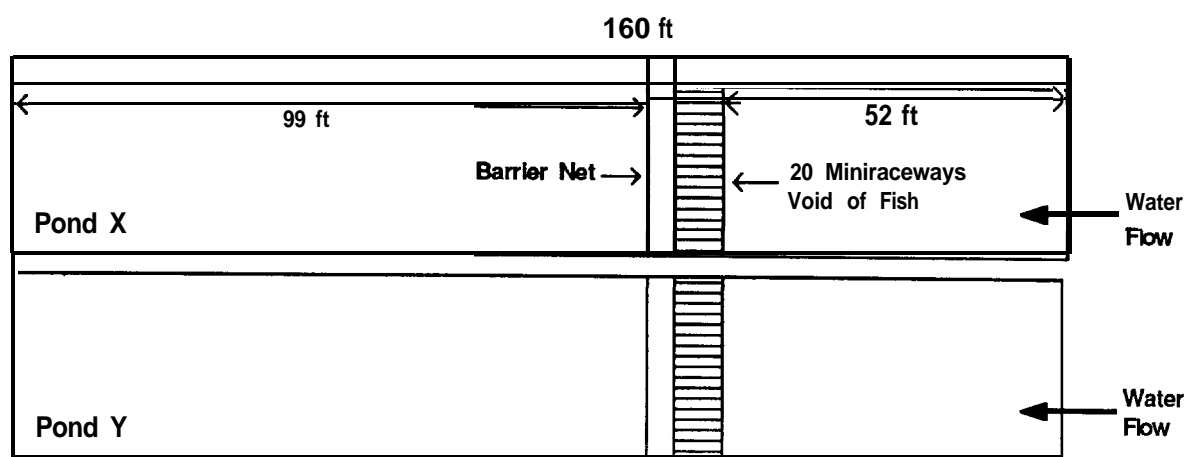
Untreated fish  
1 g size (400-450/lb)  
Raceway B in Nursery  
Odd numbered raceways  
(Total: 40,000)



Day 30 Fish moved to outside ponds  
Random placement in live boxes  
2,000 fish per live box  
4.5 g size (100 lb)  
(# live boxes: 20)

Fish moved to outside ponds  
Random placement in live boxes  
2,000 fish per live box  
4.5 g size (100 lb)  
(# live boxes: 20)

Remove 1,000 fish from each group for subsequent viral challenge in the R&D research facility.



Day 45 Sample 25 fish/live box. Pool.  
Remove to laboratory for live virus  
challenge to determine LD<sub>50</sub>.

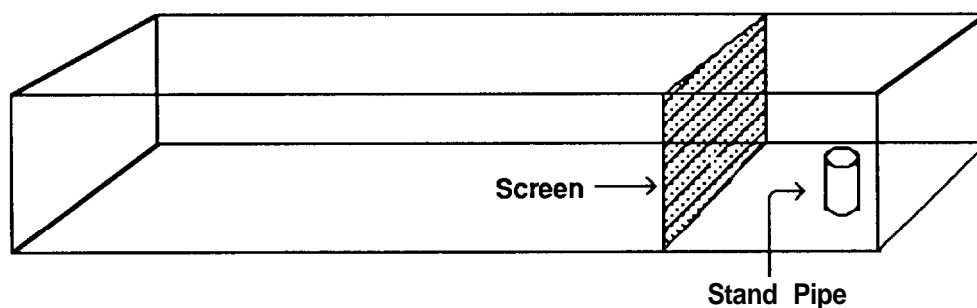
Sample 25 fish/live box as  
for vaccinated fish for virus  
challenge.

Day 60 Repeat procedures of Day 45 if no IHN outbreak has occurred among the vaccinated and untreated control fish.

## PROCEDURE FOR IMMUNIZATION

1. Fish will be immunized at 1 g (450/lb) in lots of 20,000.
2. Equipment:

Holding tank equipped with drain  
Approximate dimensions of tank: 24" W x 60" L x 18" H



3. Procedure:
  - A. 20,000 fish transferred by net into tank containing 40 gallons (152 liters) of water. (Water level noted on tank)
  - B. Water drained completely from tank.
  - C. Vaccine added [5 liters (1.32 gal) at 3 mg/ml total protein].
  - D. Fish held in vaccine for 1 min.
  - E. 50 liters (13 gal) of water added back. Pour water in with bucket. Fish held in this water for 2 min.
  - F. Water level brought to that previously noted for 40 gallons. Approximately 25.68 gallons or 97.3 liters.
  - G. Flush system to remove unabsorbed vaccine so that no vaccine enters the system.
  - H. Fish transferred by net to holding raceway and held there for 30 days.

## PROCEDURE FOR VIRAL CHALLENGE STUDIES

Task: Compare the relative resistance of fish to live virus challenge after immunization vs. no treatment.

The rainbow trout in immunized or nonimmunized groups will be challenged in the laboratory to determine the relative resistance of these two different groups of fish to IHNV. At 30 days post immunization, the fish in paired lots of 25 each will be challenged with 10,000, 1,000 and 100 TCID<sub>50</sub>/ml in a liter of water for 18 hours. After exposure to the live virus, the fish will be returned to aquaria for the duration of the test. The fish will be examined daily and dead fish will be removed and processed for IHNV isolation.

		Virus Chal. Dose			Result
		TCID <sub>50</sub> /ml*			
		10.000	1.000	100	
Sample Group	Number of Fish Per Tank				
Immunized Fish (Run in triplicate)	25	25	25	5	LD <sub>50</sub>
	25	25	25	25	Mean Death Day to
Control Fish (Run in triplicate)	25	25	25	5	LD <sub>50</sub>
	25	25	25	25	Mean Day to
	25	25	25	25	Death

- TCID<sub>50</sub>/ml = 50% tissue culture infective dose per ml

June 20, 1989

## FIELD TRIAL PROTOCOL

We fed these fish with a diet containing tetracycline for 10 days postvaccination and again at 5 days before ponding and for 10 days after ponding.

### Routine Health Monitoring:

1. Sample fish before immunization: 30 fish  
  
virus assay: 30 kidneys/spleens,  $10^{-1}$  and 1  $10^{-2}$  dilutions  
  
bacterial assay: 10 plates, 3 fish per plate, 6 sectors/plate  
gill, kidney  
streak for isolation  
  
weigh fish, compute average weight
2. Sample fish once a week during immunization period  
  
virus assay: 60 kidneys/spleens,  $10^{-1}$  and 1  $10^{-2}$  dilutions  
30 Vaccinated  
30 Control  
  
bacterial assay: 40 plates, 3 fish per plate  
gill, kidney  
streak for isolation
3. Sample fish before ponding  
  
virus assay: 60 kidneys/spleens,  $10^{-1}$  and 1  $10^{-2}$  dilutions  
  
bacterial assay: 20 plates, 3 fish per plate  
streak for isolation
4. Sample fish on day 5 and day 10 before onset of epizootic at projected day 17.  
  
virus assay: 3 fish per miniraceway, spleens and kidneys  
gills  
 $10^{-1}$  and  $10^{-2}$  dilutions  
240 fish samples x 2 dil. = 480 assays  
24 well plate, 12 fish/plate = 40 plates  
  
bacterial assay: 3 fish per miniraceway  
120 fish samples, kidneys, include gills as well  
6 fish per plate, 20 plates  
wet mounts for protozoan examination

### Epizootic Monitoring;

Every miniraceway will be treated as a separate raceway. There will be no pooling of fish for health monitoring.

1. count mortalities twice to three times a day as is practical
2. weigh fish (if large number, take average by weighing 5-10)
3. every other day (alternate between each pond)

a. Virus assays:

kidneys/spleens for each miniraceway  
monitor fish as follows:  
6 morts - individual  
7-30 morts - 6 assays and freeze individ.

Run at  $10^{-2}$  and  $10^{-4}$  dilutions in 24 well plates  
potentially  $6 \times 20 \times 2$  dil. = 240 assays/day;  
20 plates/day

b. Bacterial assays:

kidneys/spleens for each fish up to 6 fish per  
mini raceway  
Examine under microscope for protozoan infections  
6 fish per plate (TYE)  
potentially  $6 \times 20 = 120$  assays/day; 20 plates/day

4. Amend protocol as the situation warrants

#### Susceptibility to virus infection:

The fish will be challenged for susceptibility to virus infection on Day 30 (ponding date), Day 45 (15 days postponding) and, if no IHNV epizootic occurs, Day 60 (30 days postponding). On Day 30, 300 fish from the control group and 240 fish from the vaccinated group will be removed to the R&D facility at Clear Springs for live viral challenge.

On Day 45, 15 fish from each control livebox and each vaccinated livebox will be removed. The control fish will be pooled together and kept in a separate pool. The vaccinated fish will be pooled together and kept in another separate pool. The fish will then be transported to the R&D facility at Clear Springs for live viral challenge. Triplicate lots of 25 fish will be randomly removed and placed in 18 tanks per the control or vaccinated fish group. The fish will be challenged with varying dilutions of live IHN virus as follows:

Sample Group	Number of Fish Per Tank			Result
Virus Dose, TCID <sub>50</sub> /ML*	10,000	1,000	100	
		25		
Vaccinated Fish	25	25	25	LD <sub>50</sub>
	25		25	Mean Day to
	25	25	25	Death
Control Fish	25	25	25	LD <sub>50</sub>
	25	25	25	Mean Day to
	25	25	25	Death

\*TCID<sub>50</sub>/ml = 50% tissue culture infective dose per ml

RESULTS OF FIELD TRIAL  
JULY 31,1988 TO SEPTEMBER 30,1989

## RESULTS

### II. REVISED WORK PLAN - 1989 CLEAR SPRINGS TROUT COMPANY, BOX CANYON HATCHERY BUHL, IDAHO

#### Objective 4. Field Trials.

To determine the efficacy of a recombinant subunit vaccine to IHNV in field experiments at Box Canyon Hatchery, Buhl, Idaho.

The IHNV epizootics at Box Canyon Hatchery result from causes that are very different from those at Dworshak National Fish Hatchery. At Dworshak, the virus presumably enters the system in contaminated river water. IHNV positive fish spawn in the Clearwater River which provides water to the Dworshak nursery building. At Box Canyon Hatchery, the water source is spring water which is piped under the river into the nursery and the outside ponds. This water does have some fish in the headwaters of the spring; these fish presumably come from the Snake River. However, the water is thought to be virus free because there have been no IHNV outbreaks in the nursery in Box Canyon's history. The fish are exposed to the virus when they are moved from the nursery to the outside ponds. These ponds are contaminated from prior IHNV epizootics. Between different fish production lots at Box Canyon Hatchery, the outside ponds are only drained and surface scrubbed to remove any debris and algae. Immediately after these procedures, the outside ponds are refilled with the spring water and held at a constant flow rate of 6 cubic feet per second, 15° C.

The outside pond production facility is never closed down completely for full sterilization procedures before the next group of fish are put into the ponds. It is too costly for them to shut these ponds down for sterilization and there are live fish in ponds downstream from the first set of ponds used to

rear the fish transferred from the nursery (See Figure 5 for pond arrangement at Box Canyon Hatchery). Box Canyon has managed around the IHNV problem by raising more young fish. IHNV infected fish from a pond undergoing an active epizootic are often added to newly ponded fish to assure that an IHNV outbreak occurs early when the investment in raising the fish is not too large. Clear Springs Trout Company calculates projected losses to IHNV in their production schedules.

The rainbow trout in the Hagerman Valley are grown in highly oxygenated water with a constant temperature of 15° C. These conditions lead to the rapid growth of the fish. Thus, the original schedule developed for the Dworshak site was not usable at Box Canyon Hatchery. A new schedule was developed to accommodate the growth rates, development of immunocompetence, and usual occurrence of disease when fish are moved to outside ponds that was observed at Box Canyon Hatchery.

Facilities available at Clear Springs Trout Company research building were excellent. There were separate rooms equipped for diagnostic work in virology, histology, immunology, and nutrition research. In addition, there was an indoor wet laboratory equipped with specific pathogen-free water for running virus challenge studies. There was a chlorination holding tank to decontaminate the virus treated water. There were 5-gallon, 30-gallon, and 3-foot circular tanks and long rectangular troughs to hold fish in the research facility. Outside, there were individual raceways that were scaled to one third of the normal production raceway for research purposes. There was a separate entry room for infected fish to minimize contamination of the rest of the facility. Because the facilities at Clear Springs Trout

Company were so nicely equipped, we were able to run the virus challenge studies and conduct the assays on the fish mortalities there. No samples had to be shipped back to Corvallis for further studies.

Task 4.0. Obtain approval for change of site for testing the vaccine from the following offices:

Subtask 4.0.1. Dr. W. Greg Nelson, Idaho State Veterinarian.

Please see attached letter of approval.

Subtask 4.0.2. Dr. George Shibley, U.S.D.A., Veterinary Biologics Staff, APHIS, VS, 6505 Belcrest Rd. Hyattsville, Maryland 20782.

Please see attached letter of approval.

Subtask 4.0.3. Notification of Kent Hauck and Keith Johnson, Idaho Fish and Game, Eagle Fish Health Lab., Route 1, Trout Road, Eagle, Idaho 83616.

Please see attached letter informing IFG of field trial.

Task 4.1. Isolate IHNV from moribund fish at Box Canyon Hatchery.

Subtask 4.1.1. Take moribund fish from Box Canyon Hatchery to Corvallis, Oregon. Isolate virus using standard procedures.

Five moribund fish were taken out of a pond at Box Canyon Hatchery.

These fish had been diagnosed with symptoms of IHN disease by the Hatchery Biologist and Assistant Manager, Tom Lucas. Virus was isolated from all five fish and one isolate was selected at random for further study.

Subtask 4.1.2. Prepare virus stocks for shipment to Drs. Robert Busch and Sandra Ristow.

Task accomplished.

Andrew Morton at the Research Facility at Clear Springs Trout Company received the virus stock. Dr. Sandra Ristow carried out a monoclonal analysis of the Box Canyon Hatchery isolate. Please see attached Table 1 for the monoclonal reactivities of this virus isolate . The Box Canyon isolate does have a very distinct reactivity with the N-specific monoclonal antibodies. However, with the G-specific monoclonal antibodies, it was very similar to the other Hagerman Valley Clear Springs Trout Company isolate, 039-82, a Type 2 virus, and Round Butte isolate, a Type 1 virus. The Round Butte virus was the virus that was used to produce the clones from which the vaccine was derived.

Subtask 4.1.3. Prepare virus stocks for virus challenge studies.

Task accomplished.

In addition, an LD<sub>50</sub> titer of the virus stock was determined by direct assay in fish at the Clear Springs Trout Company research facility. The fish used in these assays were taken from the same egg lot as the fish that were vaccinated for the field trials. The LD<sub>50</sub> for this virus preparation was approximately 1,000 TCID<sub>50</sub>/ml in rainbow trout fry (Trout Lodge eggs) at 0.9 g (average weight). The cumulative mortality over time in days is plotted for each virus concentration used in the LD<sub>50</sub> assay in Figure 1.

Table 1. Comparison of IHNV isolates by Fluorescence Assay using Anti-IHNV monoclonal antibodies. Data generated by S. Ristow, Washington State University.

Monoclonal Antibody	Virus Isolate		
	039-82-OSU	Box Canyon	Round Butte
Anti-N MAB			
14D	+	+	+
139D	+	+	+
104Q	+	+	+
163E	+	+	+
105B	+	+	
191	+/-	++	+
18H	+		+
17w	+		+
42C			-
Anti-G MAB			
127B (IgM) neut.	+	+	+
131A (IgM) neut.	-	-	
*RB/B5 (IgM) neut.	+/-	?	+
*193-I 10 (IgG) neut.	+/-	?	+/-
151 K (IgG)	-	-	
135L (IgG)	+	+	+
136J (IgG)	+	+	+

\*The monoclonal antibodies referenced here were taken from the study by Winton et al. (1988).

Task 4.2. Vaccinate IHNV-negative fish at 1 .O g/fish (from a single egg lot) in the nursery building at Box Canyon Hatchery.

Fish were vaccinated as described in the Revised Work Plan for Field Trial of IHNV Vaccine at Box Canyon Hatchery, Buhl, Idaho on July 20, 1989. The vaccinated fish group was immunized by immersion in three 4,667 fish groups (av. wt./fish = 1 g; 10.3 lb/4667 fish). A group of 4,667 fish was put into the vaccination tank in 30 liters of hyperoxygenated water. The vaccine was measured out so that the fish received 37.5 mg total protein/100 fish for the G protein vaccine (p52G prepared at BioMed Research) and 37.5 mg/ml total protein/100 fish for the N protein vaccine (pLON-3 prepared at Oregon State University). The water was drained from the tank and the vaccine was added immediately at a total volume of 1176 mls. The fish were maintained in this concentrated vaccine mixture for 1 min and then 10 liters of water was added. The fish were maintained in the diluted vaccine mixture for 2 min. and then the vaccinated fish were returned to a separate rearing pond. This procedure was repeated two more times for a total of 14,000 vaccinated fish.

The nonvaccinated control fish were also weighed and then put into an adjacent rearing pond. It was made clear that this time the fish that were downstream from the vaccinated fish were not to be used later to make up the 200,000 unvaccinated fish that would be held downstream from the experimental fish in the outside ponds. (Previous experience with Vibrio vaccines have shown that very little vaccine is needed to immunize fish and

since we hadn't run a dose response curve on the vaccine yet, we did not want to consider this variable in our interpretation of the results.)

Scott McKibbin was hired to help build the live boxes for the outside raceways and to conduct routine health monitoring on the vaccinated and control fish. He was contracted to stay at the Clear Springs site for the duration of the experiment.

On July 28, 1989 we obtained the results of our laboratory test of the BioMed p52G vaccine and found that it did not protect fish. We called Clear Springs Trout Company and made arrangements to send someone there on August 4, 1989 (two weeks after the first vaccination) to revaccinate the same fish with p618G, a vaccine preparation which had been shown previously to be effective in the laboratory. The fish were vaccinated once again in groups of 4,667 fish (23 lbs of fish at 2.24 g/fish). The control fish remained untreated. A timeline summary of the events that occurred during the field trials is shown in Figure 2. The day the screens were removed is denoted as Day 0 for all the ensuing figures.

Task 4.3. Challenge fish by natural exposure to environmental virus in outside ponds in Box Canyon Hatchery.

On Day 29 after vaccination, August 18, 1989, the fish were transferred to the outside ponds. The average weight of vaccinated fish was 4.09 g and that of the control fish was 4.16 g. The plan called for a total of 40 miniraceways which included 20 vaccinated fish groups and 20 control fish groups of 600 fish each. Each vaccinated fish group contained 5.4 lbs of fish; each group was weighed out separately and transferred immediately to the miniraceway for a total of 20 times. For the control fish groups, 5.6 lbs of

fish were weighed out separately and transferred immediately for a total 20 times.

After the fish were distributed into the raceways, Tom Lucas (Box Canyon Hatchery Biologist and Assistant Manager) threw ten IHNV positive fish into the upper part of the outside pond. The challenge was made immediately after ponding. The construction of the miniraceways had not been completed at the time of the outside ponding because Wally MacRoberts (Clear Springs research construction) and Scott McKibbin were unable to get the job finished in time. There was some warping of the marine plywood separating each miniraceway so that some miniraceways were very wide in the middle and adjacent miniraceways were very narrow in the middle. The warping also caused some of the separating plywood dividers to lift off the bottom angle iron so that the netting was loose in the middle. That led to some of the fish swimming under the netting and dying in that space. Each miniraceway was identified by a number that was written on the top of each plywood divider. Despite these problems we never saw any mixing between control and vaccinated fish.

A group of vaccinated and control fish were transferred directly from the nursery building to the Clear Springs Research Facility wet lab. These fish were designated for tests in the laboratory to determine whether the vaccinated fish were immunized against the Box Canyon isolate of IHNV. Because we were unable to test the vaccine against the Box Canyon isolate before the initiation of these trials, a test of the vaccine under laboratory conditions was initiated in the facility. The results of this trial are shown in Figure 3. Each data point represents the average of 3 assays in vivo at the virus concentration indicated in the figure. Although protection was

evident with 15% more deaths in the control unvaccinated group at the highest virus concentration, this level of protection was low compared to the levels that have been observed with other virus isolates. Also, the fish were larger at 3.2 g/fish than is normally used in the virus challenge studies and at a virus concentration of 10,000 TCID<sub>50</sub>/ml ( $10^{+4}$ ), only 65% of the control fish were killed. When the fish were 0.9 g in size, the same virus concentration led to a 75% mortality rate. These results suggest that the vaccine formulation for the Box Canyon isolate may have to be redesigned and points out the need for adequate preparatory laboratory tests for field trials. All previous preliminary trials had been designed for Dworshak National Fish Hatchery.

A third laboratory challenge trial was conducted on these fish on September 8, 1989. One set of control and vaccinated fish which had been held in the wet lab under specific pathogen-free water conditions was challenged with 1000 TCID<sub>50</sub>/ml Box Canyon IHNV. The average weight of fish in this group was 7.10 g. Another set of vaccinated and control fish was obtained from the miniraceways at Box Canyon Hatchery and brought into the containment facility at the Clear Springs research facility. The average weight of these fish were 7.6 g. These fish were divided into two groups of three tanks each. One group of Box Canyon vaccinated fish received 1000 TCID<sub>50</sub>/ml Box Canyon IHNV and another group of Box Canyon vaccinated fish received 10,000 TCID<sub>50</sub>/ml Box Canyon IHNV. A similar challenge regime was used on the Box Canyon control group. The results of these laboratory trials are shown in Figure 4. No protection was exhibited by the vaccinated group of fish. The loss of protection after six

weeks was completely different from results obtained in previous trials in the laboratory with other virus isolates such as Dworshak 1984 and 1988, Round Butte 1, and Cedar River.

#### Results of Field Trials Exposure of Fish to IHNV-

The fish were transferred to the outside ponds on September 18, 1989 and two weeks later, there was no outbreak of IHNV among the 200,000 fish downstream from the miniraceways in each of the two outside ponds at Box Canyon. Concern was expressed by the Box Canyon Hatchery management that an outbreak should be induced soon or the fish might escape an epizootic episode during this period of growth. Management did not want these fish to break with IHNV later. Thus, 3000 fish from the downstream section of each pond were transferred to the top of each pond. After five days, when there was still no outbreak of IHNV in these ponds, Tom Lucas (Box Canyon Hatchery Biologist and Assistant Manager) added 20 IHNV positive fish to the top of each pond. On September 12, 1989, when there was still no outbreak, the screens separating the unvaccinated 200,000 fish from the miniraceways were removed and infected fish were once more added to the top of each pond. This date has been set as Day 0 in Figure 2. It is really 25 days after ponding. Finally, on September 18, 1989 (one month after ponding), the fish in one pond began showing signs of an IHNV epizootic.

The cumulative mortalities for the control and vaccinated fish in Pond Y (See Figure 5 for diagram of pond arrangement) are shown in Figure 6. There was a delay in the onset of mortalities in the vaccinated fish by 8 days. On September 26, 1989, fish dying of IHNV were observed among the vaccinated fish and within a week, the cumulative mortalities among the vaccinated fish

reached that of the control fish. The most likely explanation for these results was that the immunoprotective response induced in the vaccinated fish had been overwhelmed by the high concentration of virus produced by the 200,000 unvaccinated and IHNV-infected fish surrounding the miniraceways. Fish dying of IHN disease began to show up in this population in large numbers by Day 5 (Figure 8). Laboratory trials had shown that the immunoprotective response could be overwhelmed by high concentrations of IHNV (See Engelking and Leong, 1990 and Appendix item 8.). A concern that the experimental design did not account for this fact had been brought up in early discussions and separating screens had been added to the design for this reason. It is clear that when the mortalities in the entire pond are plotted as percent mortality with that of the data from the control and vaccinated fish in the miniraceways, the virus released by the fish surrounding the raceways led to the deaths seen in the control fish and ultimately, the virus concentration reached levels that overwhelmed the immunoprotective response of the vaccinated fish (Figure 9).

A more detailed analysis of the data indicating the cumulative mortalities found in each miniraceway on Days 10, 12, 18 and 24 is shown in Figures 7A, 7B, 7C and 7D. On Day 10, it is clear that fish are only dying in the control miniraceways. By Day 12, mortalities are beginning to appear in the vaccine miniraceways and by Day 18, the peak mortalities have been reached. There are no substantial differences between Day 18 and Day 24.

A summary of the important events that occurred during this field trial are noted in Figure 2. The fish above each date indicates those times when IHNV infected fish were added to the top of each pond.

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## LEGENDS

Figure 1. Determination of LD<sub>50</sub> titer for the Box Canyon IHNV isolate in 0.9 g rainbow trout fry. The cumulative percent mortality on the ordinate is plotted against the number of days after virus challenge. Each point represents the average percent mortality of four replicate tanks, 104 fish per tank. The symbols indicate virus concentrations of  $10^1 = 10$ ,  $10^2 = 100$ ,  $10^3 = 1000$ , and  $10^4 = 10,000$  TCID<sub>50</sub> units per ml of virus.

Figure 2. Time line representation of events that occurred during the field trial of the IHNV vaccine.

Figure 3. Graphic representations of the comparison between vaccinated and control fish susceptibility to Box Canyon IHNV challenge. The fish had an average weight of 3.2 g. The virus challenge occurred one month after vaccination. Each point represents the average cumulative percent mortality for three replicate tanks of 42 fish.

3A. Comparison of the LD<sub>50</sub> titer of Box Canyon IHNV in vaccinated and control fish.

3B. Cumulative percent mortality of control fish challenged with three different virus concentrations:  $10^2 = 100$ ,  $10^3 = 1000$ , and  $10^4 = 10000$  TCID<sub>50</sub> units per ml.

3C. Cumulative percent mortality of vaccinated fish challenged with three different virus concentrations:  $10^2 = 100$ ,  $10^3 = 1000$ , and  $10^4 = 10000$  TCID<sub>50</sub> units per ml.

3D. A comparison of vaccinated and control fish susceptibility to Box Canyon IHNV at a concentration of  $10^2$  or 100 TCID<sub>50</sub> units per ml.

3E. A comparison of vaccinated and control fish susceptibility to Box Canyon IHNV at a concentration of  $10^3$  or 1000 TCID<sub>50</sub> units per ml.

3F. A comparison of vaccinated and control fish susceptibility to Box Canyon IHNV at a concentration of  $10^4$  or 10000 TCID<sub>50</sub> units per ml.

Figure 4. Graphic representations of the comparison between vaccinated and control fish susceptibility to Box Canyon IHNV challenge. The fish had an average weight of 7 g. The virus challenge occurred 60 days after vaccination. Each point represents the average cumulative percent mortality for three replicate tanks of 28 fish.

4A. A comparison of vaccinated and control fish susceptibility to Box Canyon IHNV at a concentration of  $10^{+3}$  or 1000 TCID<sub>50</sub> units per ml. These fish were taken from the miniraceways and transferred to the Clear Springs Trout Company Research wet lab.

4B. A comparison of vaccinated and control fish susceptibility to Box Canyon IHNV at a concentration of  $10^{+4}$  or 10000 TCID<sub>50</sub> units per ml. These fish were taken from the miniraceways and transferred to the Clear Springs Trout Company Research wet lab.

4C. A comparison of vaccinated and control fish susceptibility to Box Canyon IHNV at a concentration of  $10^{+3}$  or 1000 TCID<sub>50</sub> units per ml. These fish were taken directly from the nursery building at Box Canyon Hatchery one month after vaccination and held in the Clear Springs Trout Company Research wet lab for another month before this challenge was initiated.

Figure 5. Diagram of the facilities at Box Canyon Hatchery

5A. General layout of Box Canyon Hatchery

5B. Arrangement of the miniraceways in Pond Y. Each miniraceway contained 600 vaccinated (V) or control (C) fish. The divider screens were removed on September 12, 1989 to permit the 200,000 unvaccinated fish free access to the headwaters of Pond Y.

Figure 6. Graphic representation of the cumulative mortalities in the miniraceways of Pond Y. Each point represents the average of the 10 control or the 10 vaccine raceways present in Pond Y.

Figure 7. Comparisons of the cumulative mortalities of control versus vaccinated fish miniraceways. The abscissa shows the miniraceway number as shown in Figure 5.

7A. A comparison on Day 10.

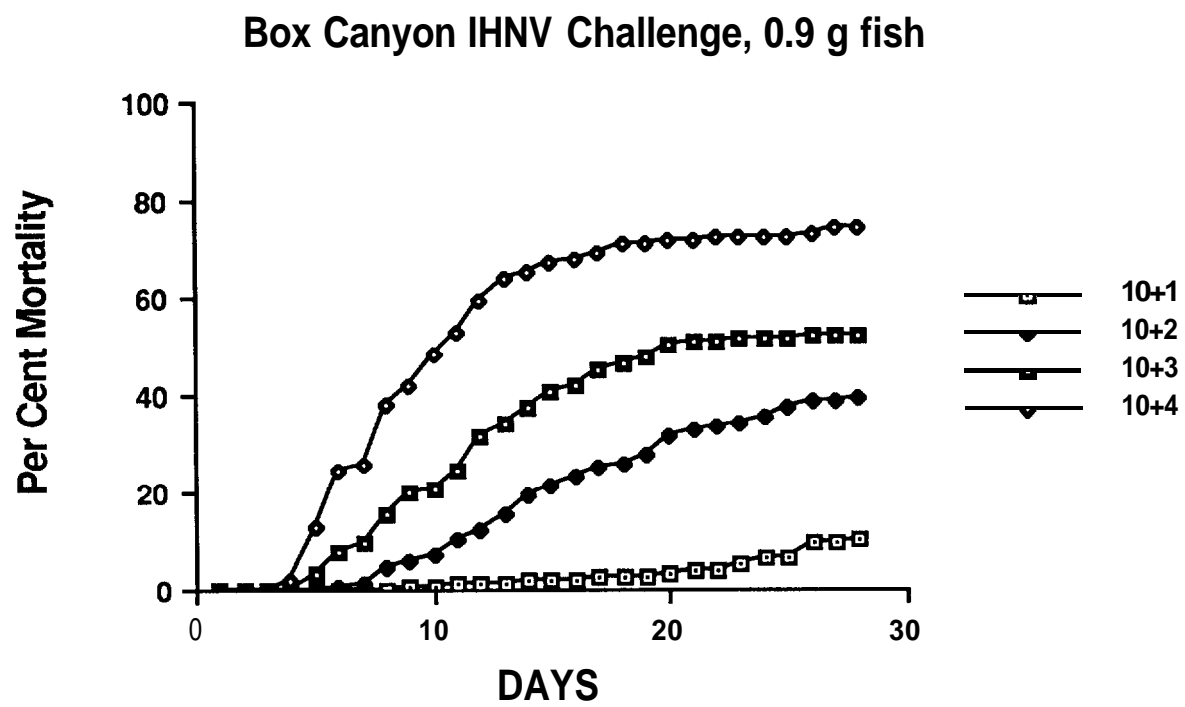
7B. A comparison on Day 12.

7C. A comparison on Day 18.

7D. A comparison on Day 24, the end of the field trial.

Figure 8. Cumulative mortalities in Pond Y among its 200,000 unvaccinated fish population.

Figure 9. Comparison of the percent cumulative mortalities of vaccinated, control and Pond Y (200,000 fish) fish.



✱

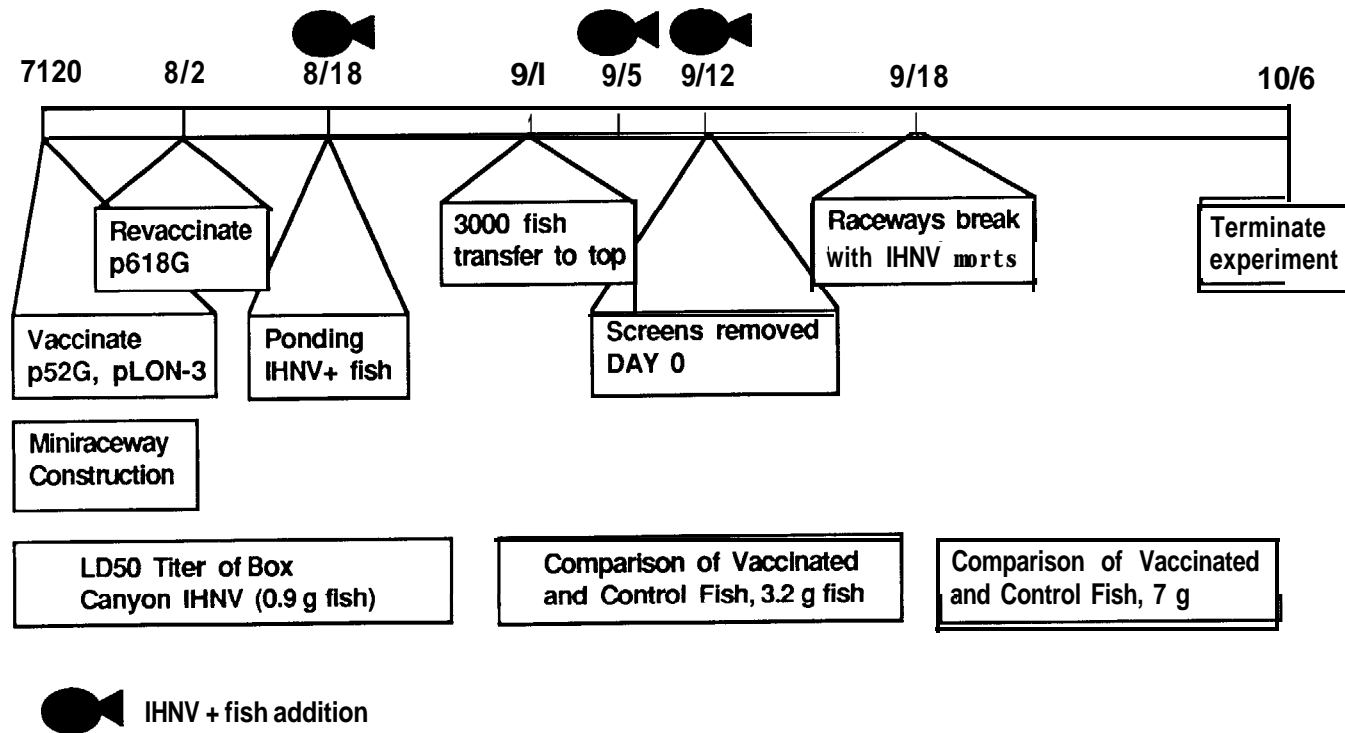
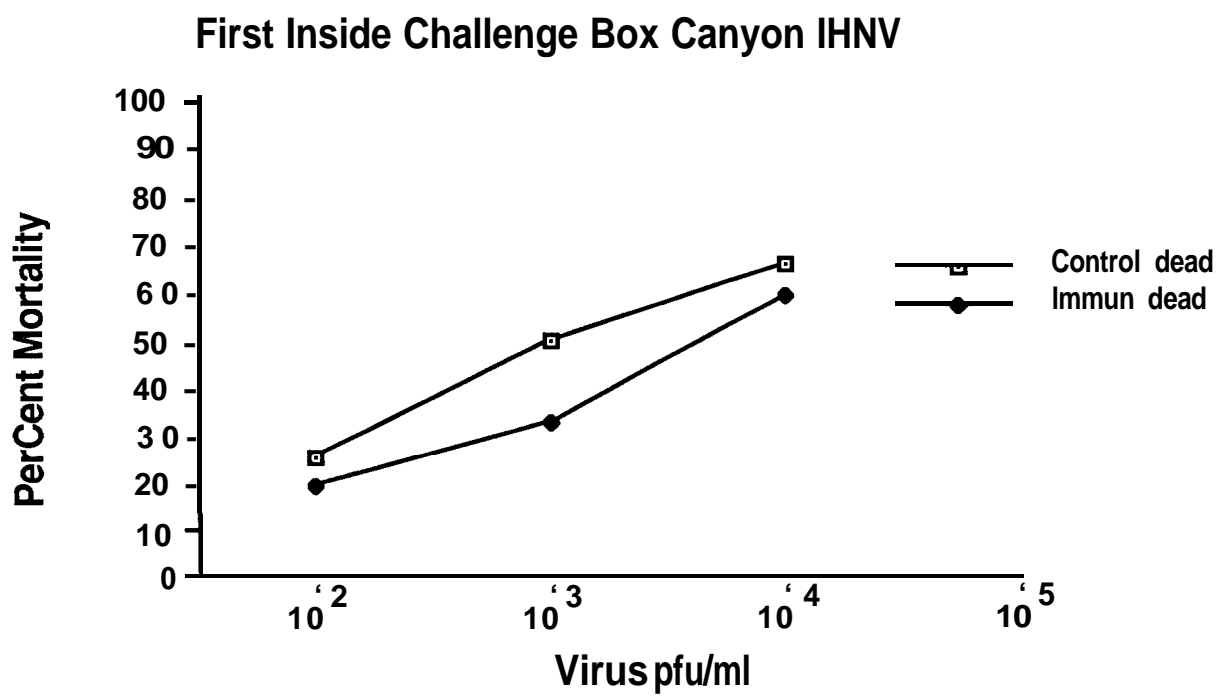
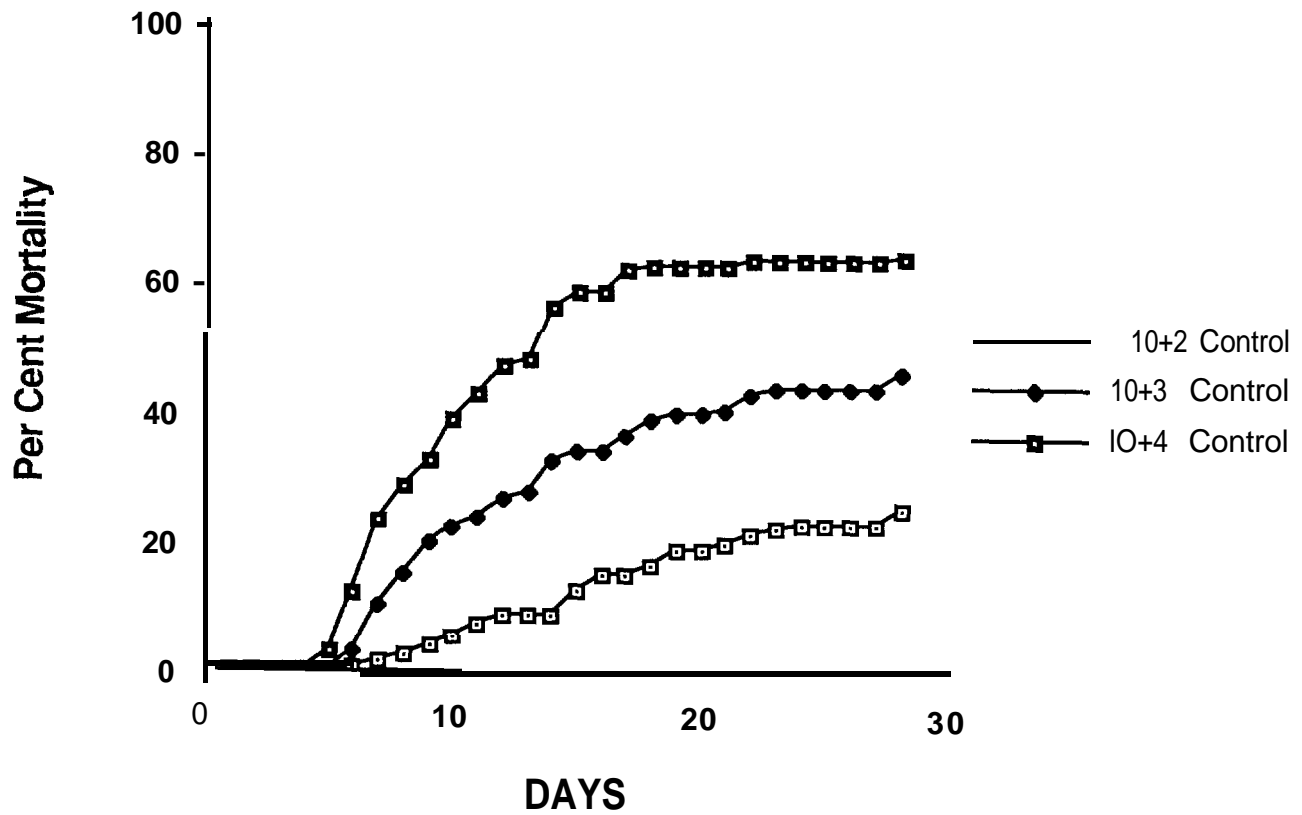


Figure 2



### Box Canyon Challenge, Controls, 3.2 g fish



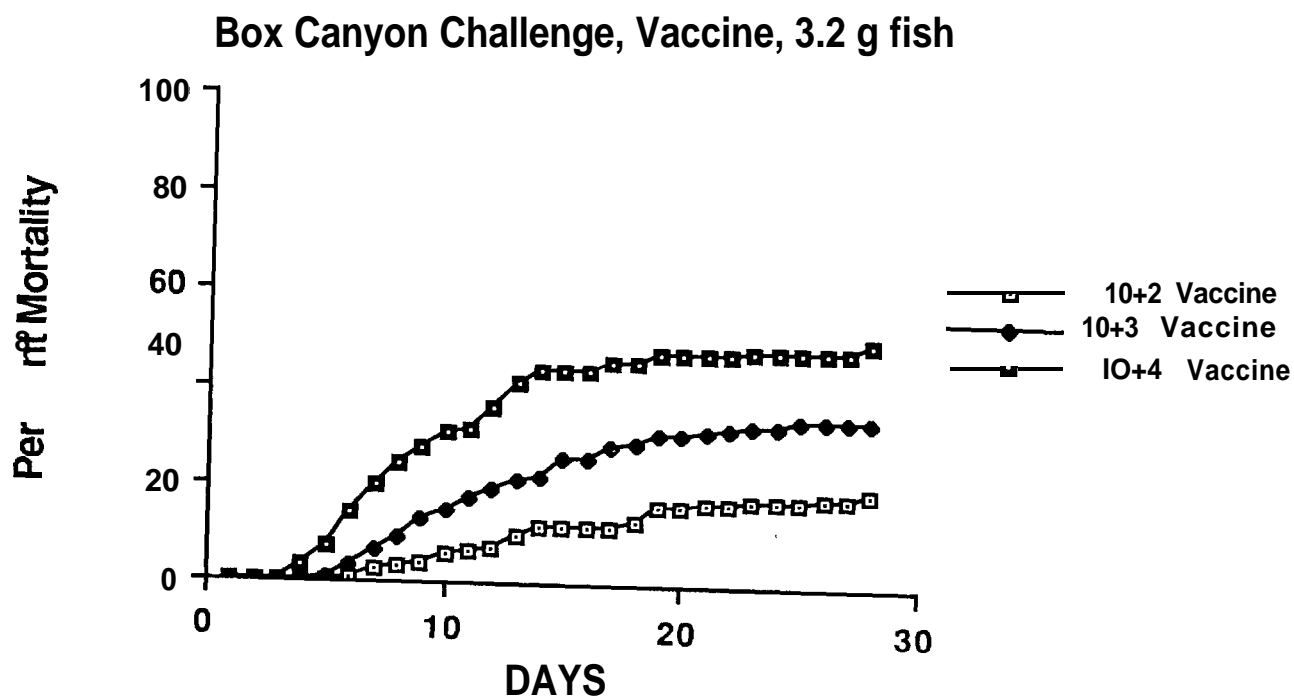
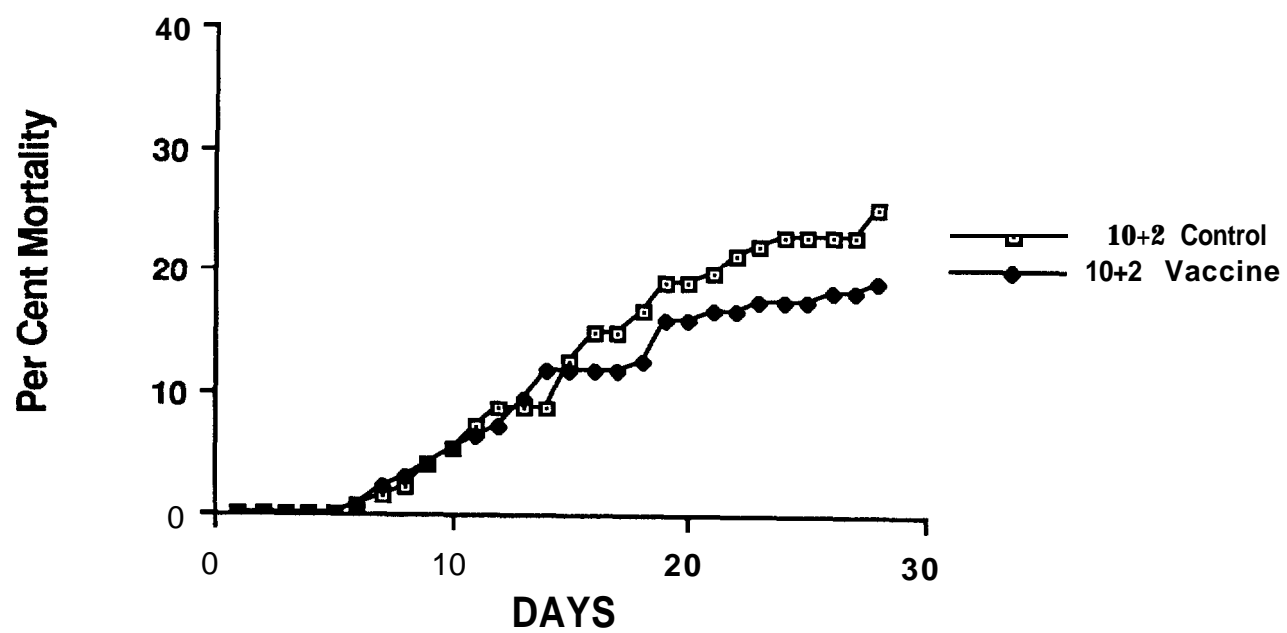
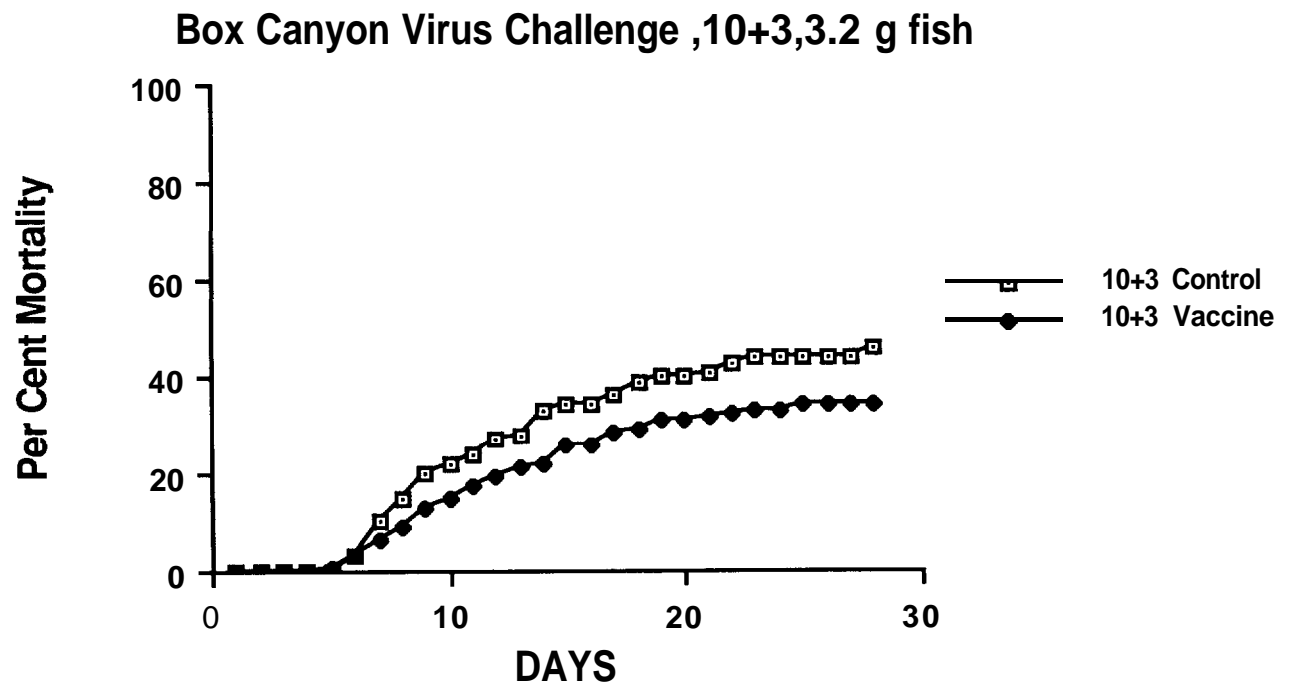
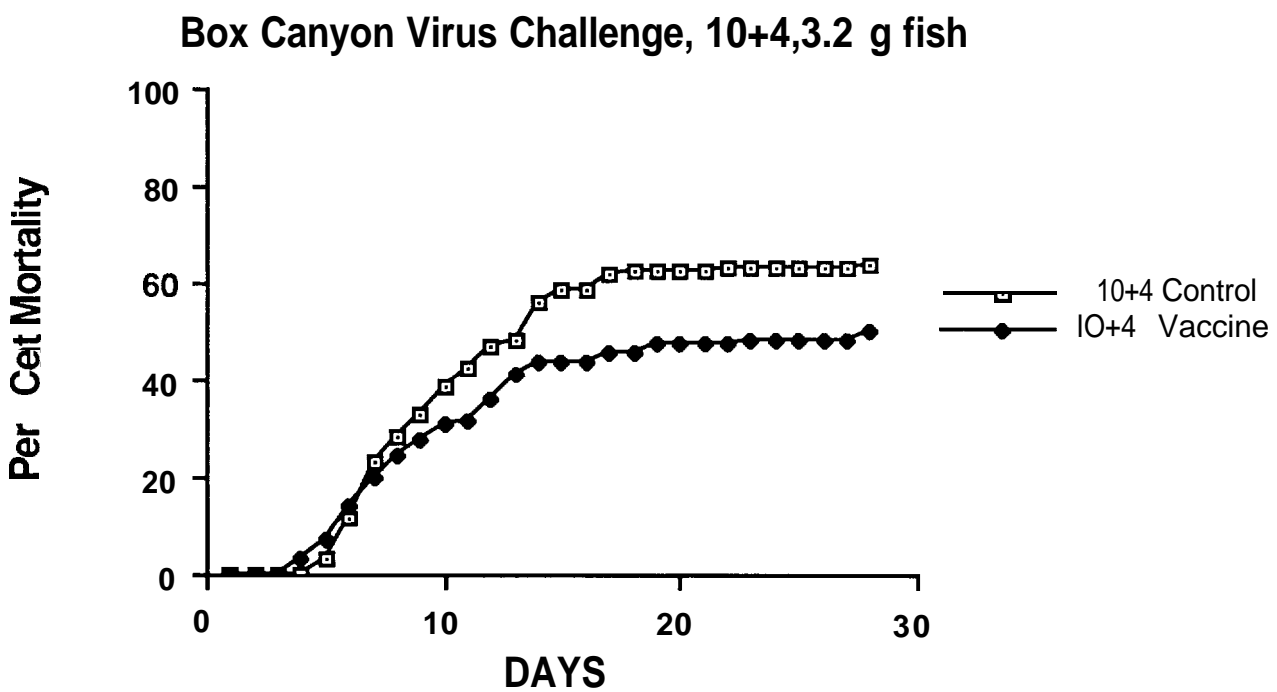


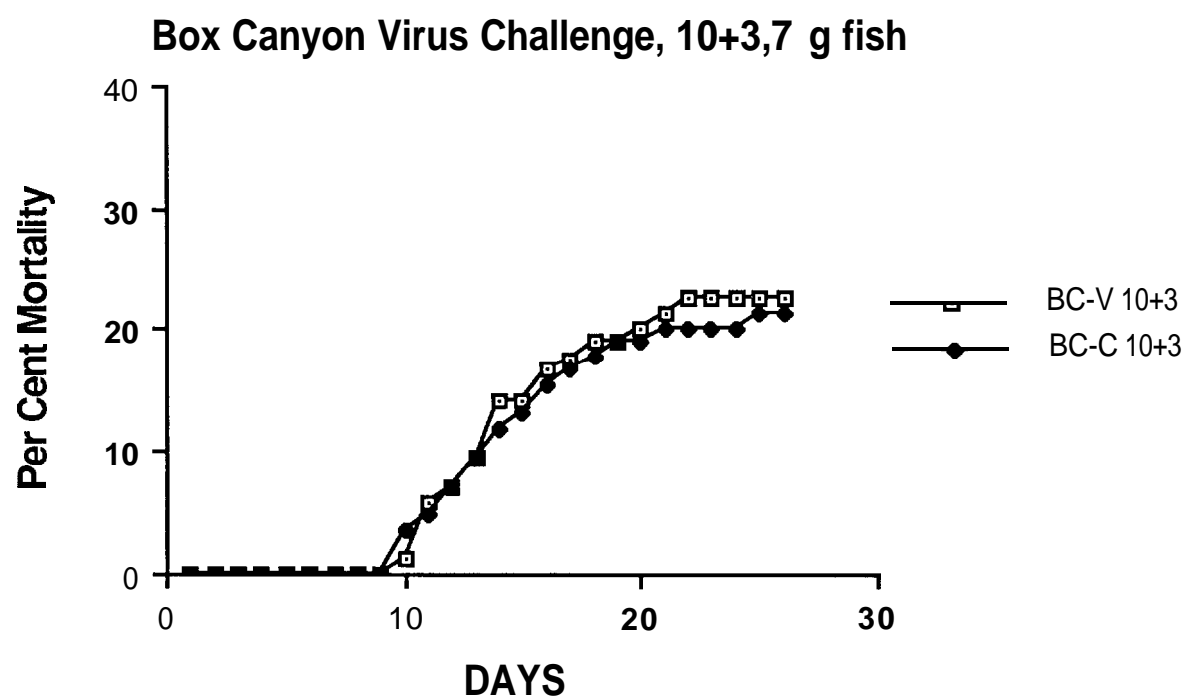
Figure 3C.

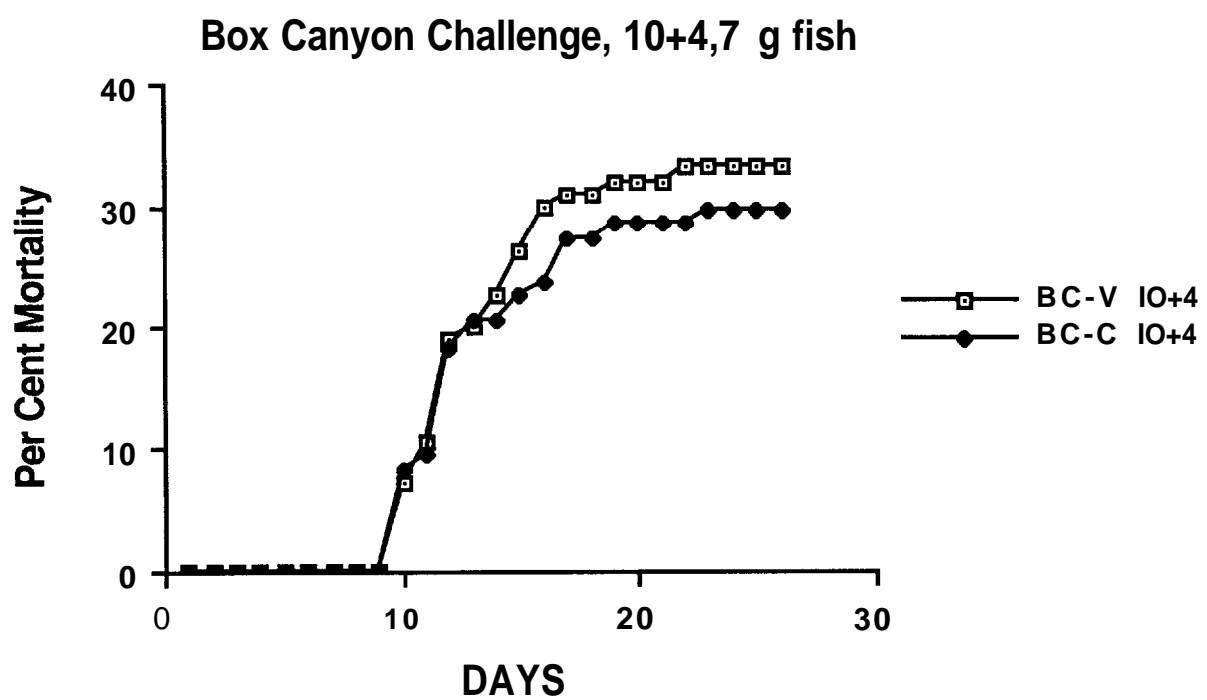
### Box Canyon Virus Challenge, 10+2,3.2 g fish

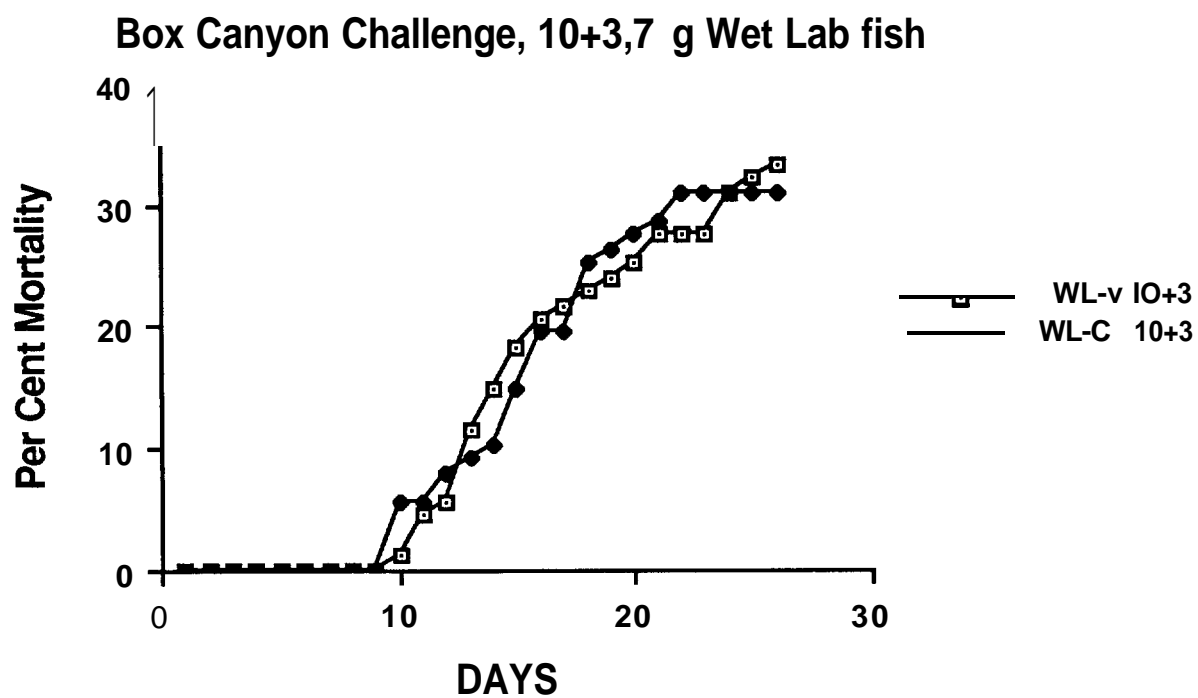




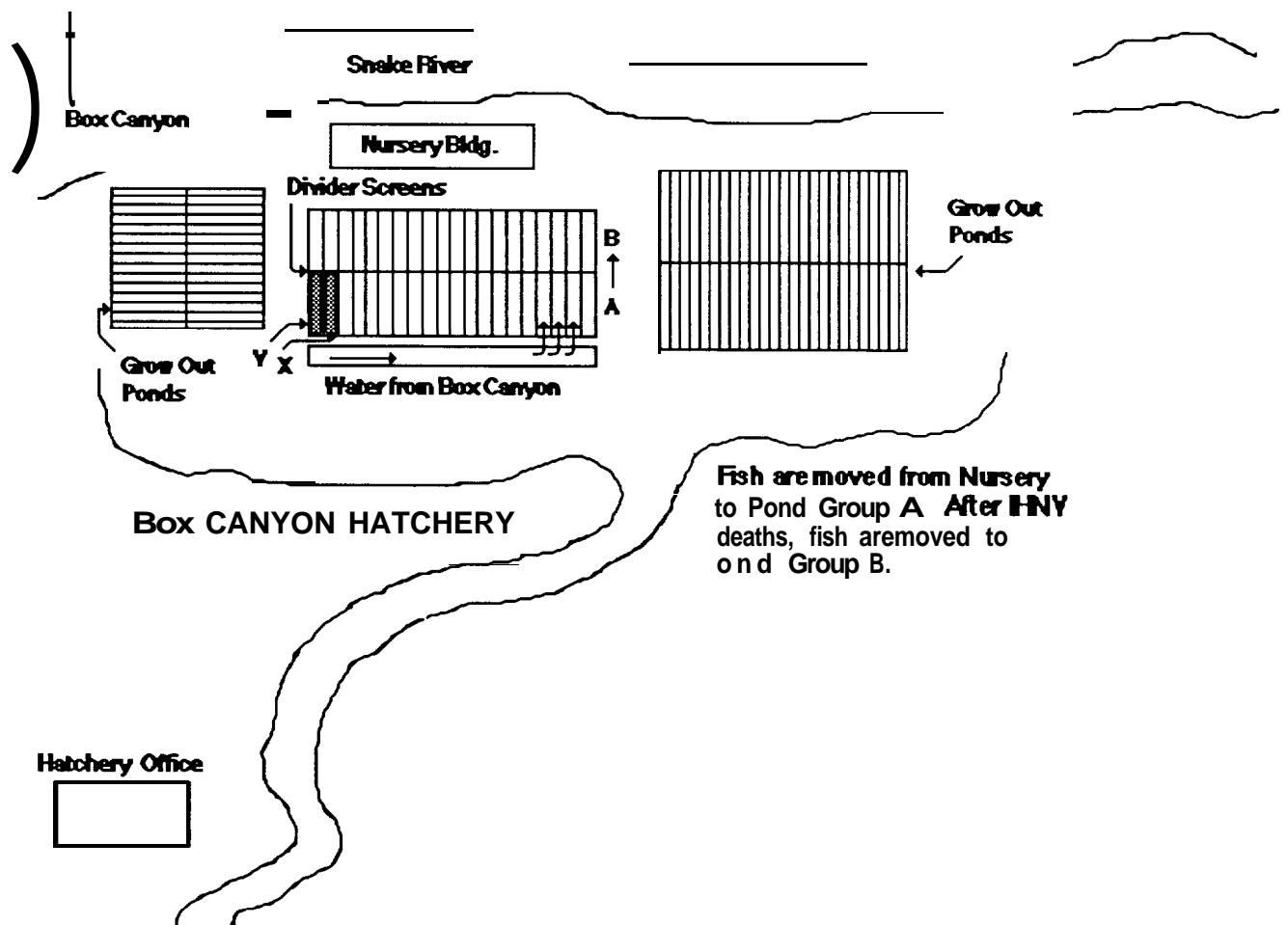


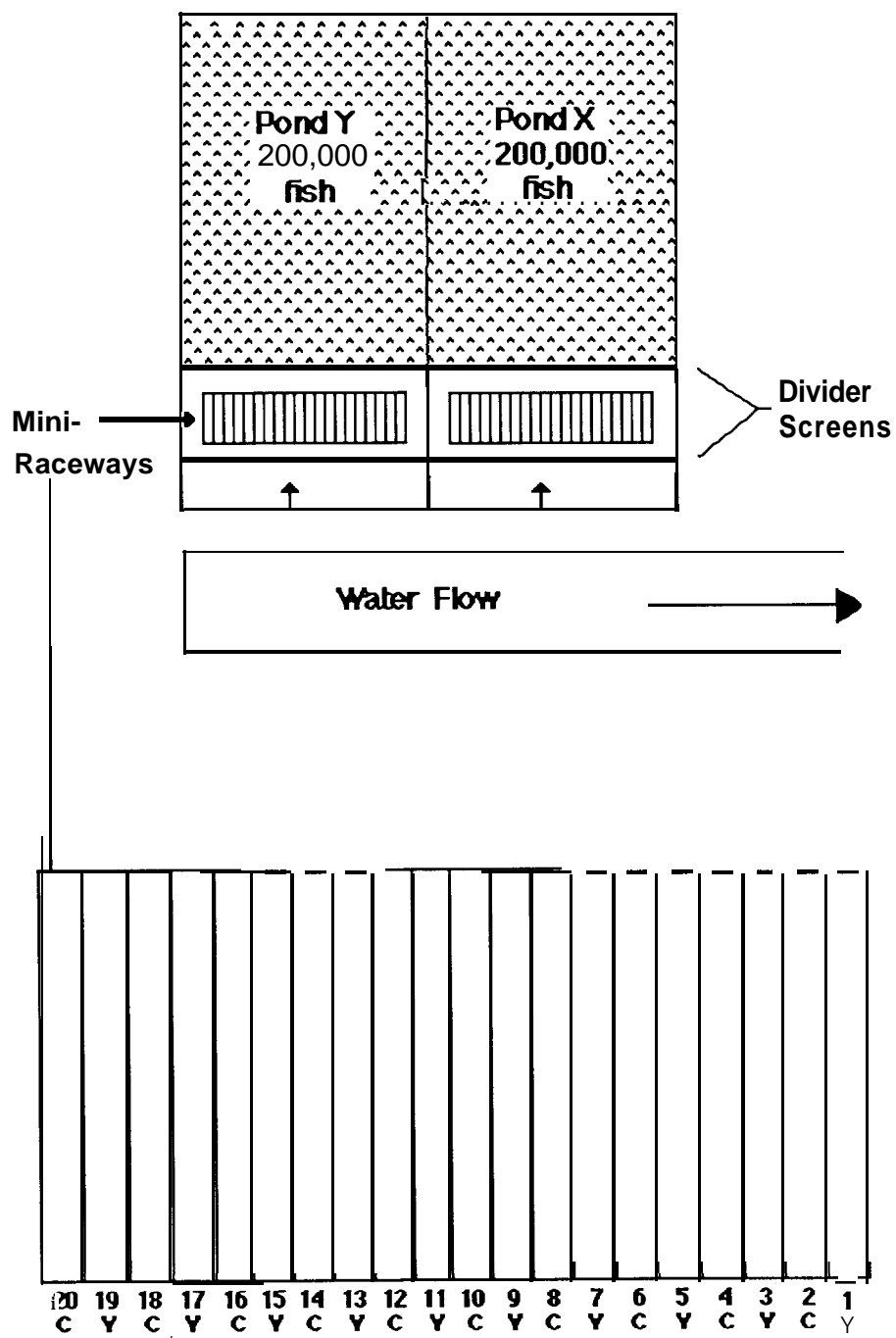




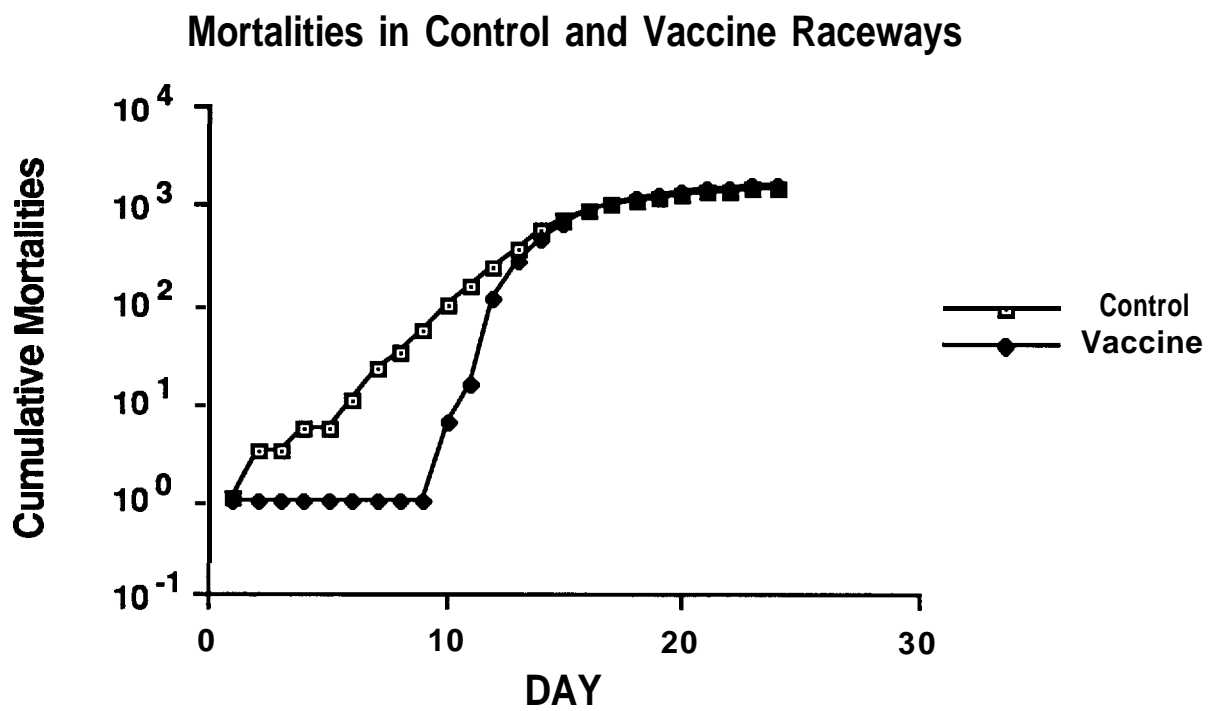


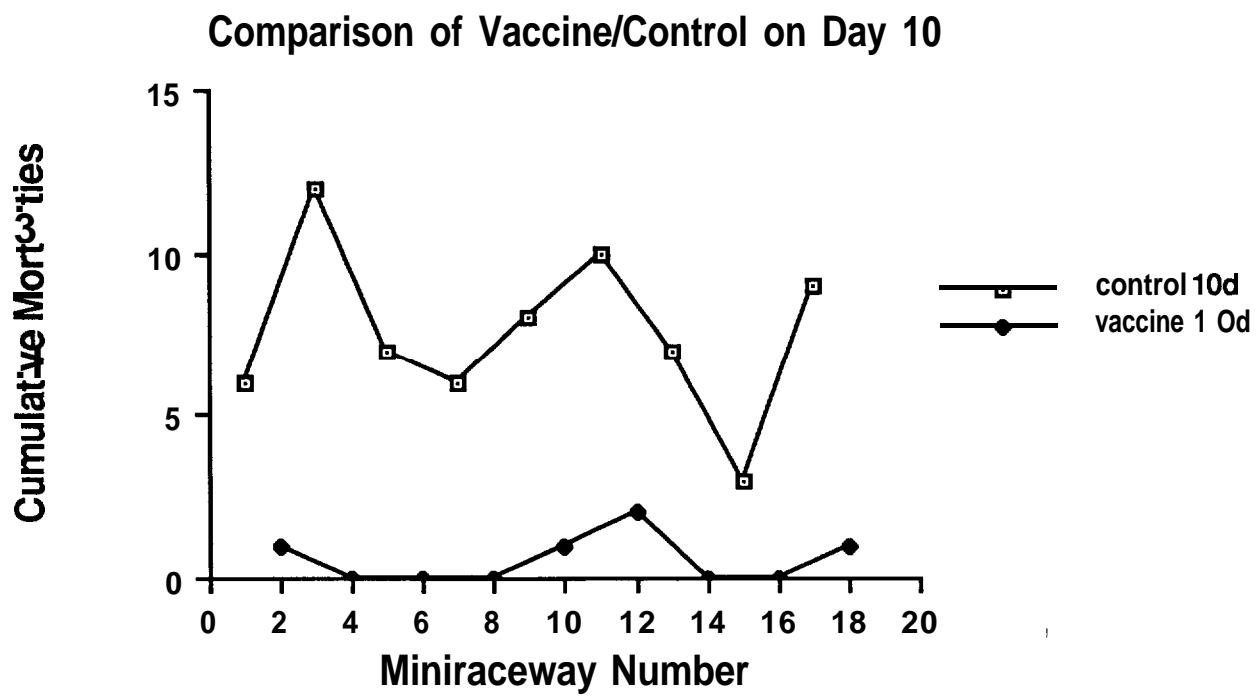
Spring source



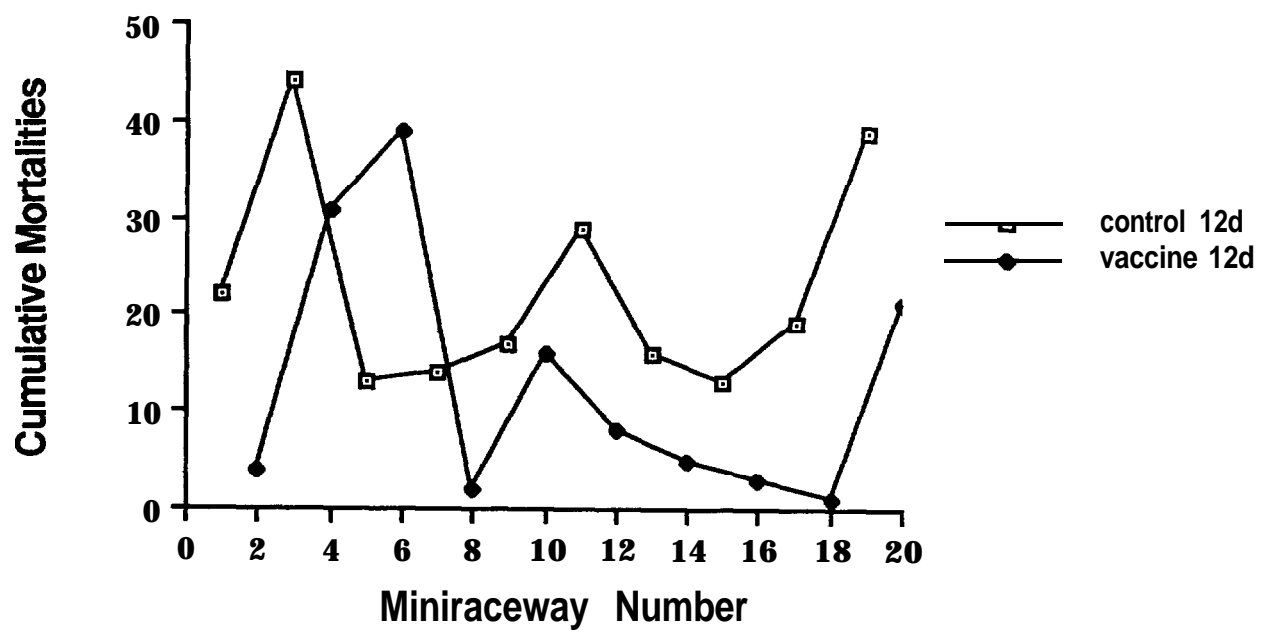


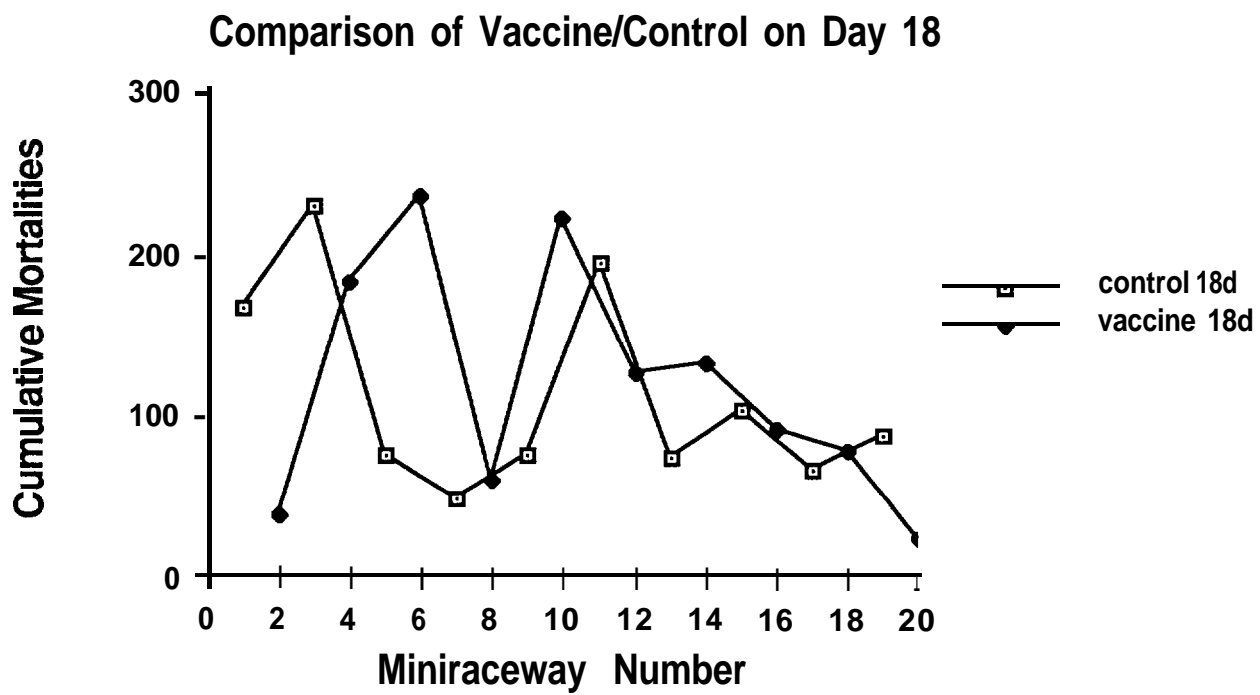
**ENLARGED VIEW OF MINIRACEWAY Y SHOWNG  
NUMBERING SYSTEM**

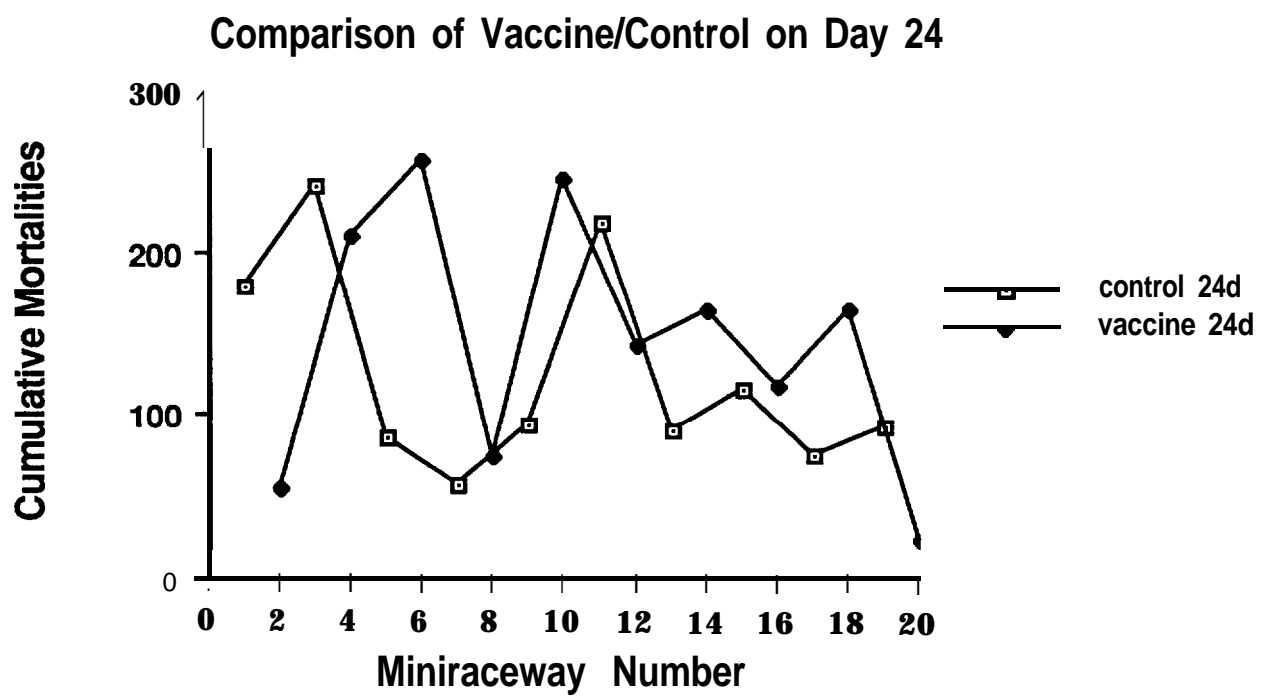


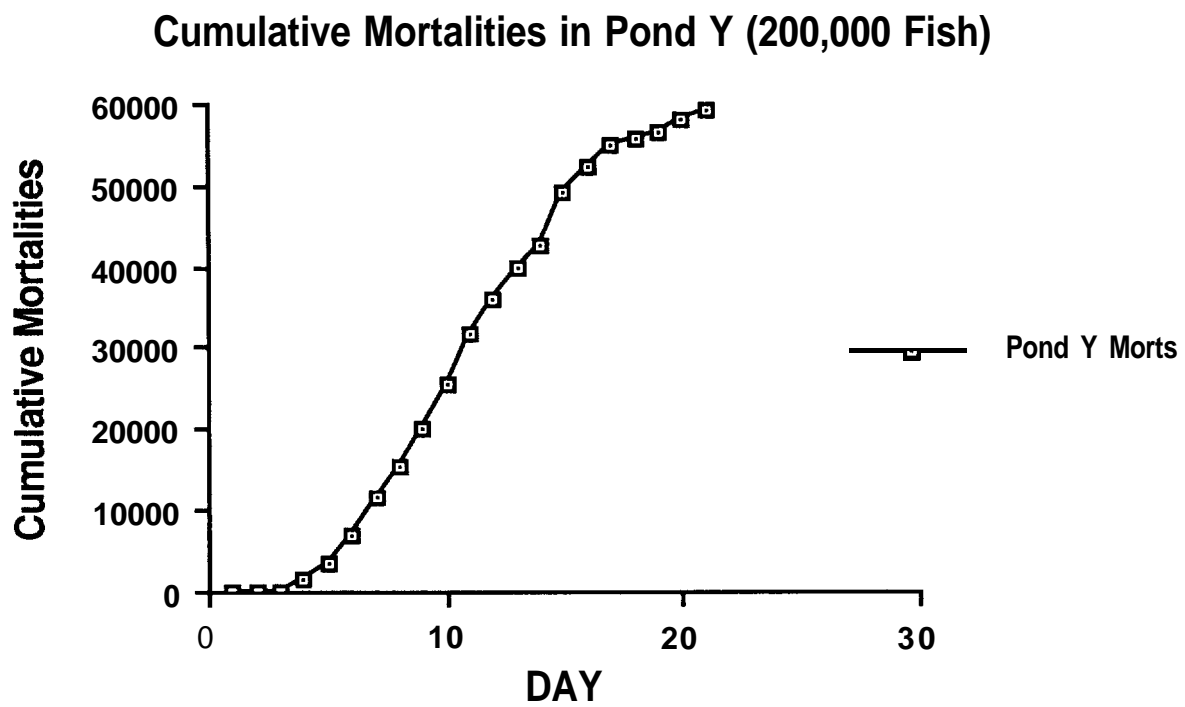


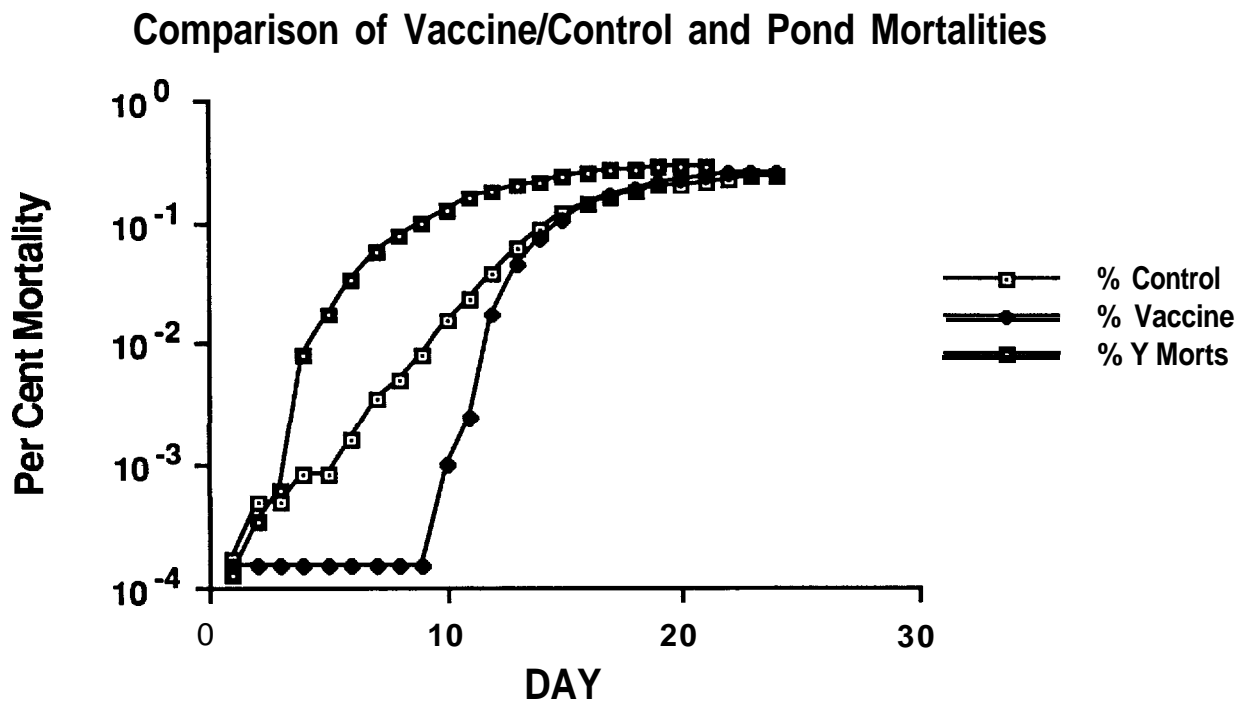
Comparison of Vaccine/Control on Day 12











## APPENDICES

## PROCEDURES

### Virus Assays:

1. Prepare six 24-well plates from 1 EPC flask (150 cm), should take 12-24 hr to come to 80-90% confluency. (During epizootic, need 20 plates per day).  
Must have available 4 EPC flasks per day  
Scott LaPatra, ODFW, prepares plates in the following manner:  
Remove media, wash cells with TBS  
Add trypsin to disaggregation (3 mls/flask)  
Add 120 mls media containing Tris buffer  
Plate 0.5 ml cells/well (72 mls for 6 plates)  
Leave remaining cells in flask, ready in a week at RT (check on growth--may be sooner)  
Need 28 EPC flasks per week to rotate so that there are 4 flasks available every day
2. Remove kidneys/spleens (everything kept on ice)  
Weigh tissue  
Add 10 times TBS (w/v) 1 :10 dilution  
homogenize in stomacher  
remove to 17 x 100 cm Falcon plastic tube  
spin 2000 x g for 10 min  
Remove supernatant and add anti-INC  
add 200  $\mu$ l to 1.8 ml anti-INC now 1 :100 dilution  
Anti-INC conc. = 1000  $\mu$ g/ml gentamicin  
500 IU/ml fungizone  
incubate 2 hr at 15° C or overnight at 4° C.  
Pretreat monolayers with 7% PEG 10  $\mu$ l/well  
make PEG in antibiotic media  
do not autoclave  
use a repeating pipettor  
Assay 100  $\mu$ l extract/well  
1 :100 dilution (10  $\mu$ l/90  $\mu$ l TBS) (1 0<sup>-4</sup> dil.)  
use a round bottom multi-titer plate to do dilutions  
Put on shake platform for 1 hour  
Assay 100  $\mu$ l/well  
Overlay with methylcellulose (0.5 ml/well)  
Read in 6-8 days

### Bacteriological Assays:

1. Streak for isolation, kidney tissue  
TYE plates  
Identify dominant colonies as in Blue Book (see attached)  
Gram S t a i n

## CALCULATIONS FOR SUPPLIES

Week 1	3 x 24 well plates 1 flask EPC cells 10 TYE plates
Week 2	6 x 24 well plates 1 flask EPC cells 20 TYE plates
Week 3	6 x 24 well plates 1 flask EPC cells 20 TYE plates
Week 4 (ponding)	6 x 24 well plates 1 flask EPC cells 20 TYE plates
Week 5 (day 5 postponding)	40 x 24 well plates 7 flasks EPC cells 20 TYE plates
Week 6 (day 10 postponding)	40 x 24 well plates 7 flasks EPC cells 20 TYE plates
Week 7 (epizootic should start)	140 x 24 well plates 24 flasks EPC cells 140 TYE plates
Week 8	140 x 24 well plates 24 flasks EPC cells 140 TYE plates
Week 9	140 x 24 well plates 24 flasks EPC cells 140 TYE plates
Week 10	140 x 24 well plates 24 flasks EPC cells <b>140 TYE plates</b>

## FIELD TRIAL SUPPLY LIST

### Disposable Supplies:

Tissue culture - 24 well plates	661
Stomacher bags	1190
Centrifuge tubes	2380
Anti-INC	2142 ml
Crystal violet stain and formaldehyde fixing solution	500 ml
Hanks balanced salt solution	5 liters
RPMI for cell growth (5% fetal calf serum)	
330 mls for 24 well plates	5 liters
4560 mls for cell splits	
Tris-buffered MEM-5	1 liter
Methylcellulose	330 ml
EPC cells in 150 cm <sup>2</sup> flasks	30 flasks
150 cm <sup>2</sup> flasks	30 flasks
1 xTBS	6610 ml
Trypsin	500 ml
Pipets 25 ml	250
10 ml	1000
1 ml	500
Pipet tips	
10 holders	
2 pipetman, 200 µl	
bag of pipet tips	

### Bacteriologic assays

Plates (6 assays per plate)	670 plates
Media TYE	8 liters
Inoculating needles	6 loops
(possibly toothpicks, autoclaved)	
Scalpels	6
Tweezers	6
Surgical scissors	6
Scalpel blades	1 box
Alcohol burners	2



# STATE OF IDAHO

DEPARTMENT OF AGRICULTURE  
DIVISION OF ANIMAL INDUSTRIES

CECIL D. ANDRUS  
*Governor*  
RICHARD R. RUSH  
*Director*

2270 Old Penitentiary R  
P.O. Box 7249  
Boise, Idaho 83707

(208) 334-3256

7 July 1989

Dr. JoAnn C. Leong  
Department of Microbiology  
Oregon State University  
Nash Hall 220  
Corvallis OR 97331-3804

Dear Dr. Leong;

Permission is hereby granted to conduct field trials on IHN vaccine (Killed Virus) at Clear Springs Trout, Buhl, Idaho. Trials will be per protocol filed with the Idaho Dept. of Agriculture and will be under the direction of Dr. Bob Busch.

This permission is given with the stipulation that the trial is also cleared by USDA, APHIS Veterinary Biologics Division.

Sincerely:

A handwritten signature in dark ink, appearing to read "W.G. Nelson".

W.G. Nelson, Administrator  
Idaho Division of Animal Industries

cc: Dr. Bob Busch  
Dr. Cecil Watson



United States  
Department of  
Agriculture

Animal and  
Plant Health  
Inspection  
Service

Biotechnology,  
Biologics, and  
Environmental  
Protection

July 20, 1989

Dr. Jo-Ann C. Leong  
Professor of Microbiology  
Department of Microbiology  
Oregon State University  
Nash Hall 220  
Corvallis, OR 97331-3804

Dear Dr. Leong:

This is in reply to your facsimile transmissions and submissions of July 11 and 18, 1989, concerning the field testing of your recombinant DNA vaccine for Infectious Hematopoietic Necrosis (IHN) Virus.

This will confirm verbal authorization of July 17, 1989, to conduct these trials with the inactivated experimental product in Idaho where written permission from State regulatory authorities has been filed. The test will be conducted in accordance with your filed procedure. This authorization is valid for 1 year from the date of issuance. A summary of the results must be filed with this office.

Sincerely,

George P. Shib/ley, Ph.D.  
Senior Staff Microbiologist  
Veterinary Biologics  
Biotechnology, Biologics,  
and Environmental Protection



Department of  
Microbiology



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Corvallis, OR 97331-3804 USA

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osu covs  
OSU FAX: 503-754-2400

July 10, 1989

Kent Hauck and Keith Johnson  
Idaho Dept. of Fish and Game  
Route A, Trout Road  
Eagle, ID 83616

Dear Kent and Keith,

I am sending you a copy of the revised workplan for field testing of the vaccine for IHNV. It has been revised for testing at the Box Canyon Hatchery in Buhl, Idaho. The contact person at the hatchery is Dr. Robert Busch, Director of Research and Development, Clear Springs Trout Company.

We are seeking to begin the immunizations on July 19, 1989 and we should be able to keep all studies on site. If there are any questions or if I can send you further information, please call me at Oregon State University.

I have appreciated your help on developing the appropriate protocols for these trials. I am trying to obtain enough vaccine for you to try and will keep you informed about any other developments. Best regards.

Yours truly,

A handwritten signature in cursive script, appearing to read "Jo Ann", written over the typed name.

Jo-Ann C. Leong, Ph.D.  
Professor of Microbiology

JCL:rd

Encl.

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## IHNV DWORSHAK CHALLENGE OF G-FUSION IMMUNIZED FISH

